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(54) Title of the Invention: Method of Analyzing DNA Segments that have been marked with Modified Ribonucleotides Where at least One Terminal can be Identified Using Associated Molecules

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Scope of Patent Claims

1. Modified DNA of the sort that does not interfere with the ribonucleotides that bond at the terminals of the DNA. This DNA has been modified using a modified oligomer, preferably a unique modified ribonucleotide that has been bonded to at least one terminal. The modification of the ribonucleotide itself is made up of chemical molecules that are covalently bonded to the aforementioned ribonucleotides. The aforementioned chemical molecules contain at least one base that does not participate in the aforementioned covalent bond and the base described above has a specific affinity for the base described above. This makes it possible for molecules or substances that can recognize the aforementioned base to bond to it either directly or indirectly. Moreover, the ribonucleotides that bond at the terminals of the DNA contain the aforementioned base in the presence of terminal DNA transferase when the ribonucleotides come into contact with the DNA under conditions where at least 1 ribonucleotide can bond to a DNA terminal.

2. The modified DNA described in Claim 1 of the range of claims with the following characteristics. The aforementioned modified base can be specifically recognized directly using a separate molecule or substance that itself can be easily detected using visual means.

3. The modified DNA described in Claim 2 of the range of claims with the following characteristics. The modified bases above are made up of antibodies or hapten and they can be identified using the antibodies that are formed in advance, in response to the respective antibodies or hapten described above.

4. The modified DNA described in Claim 1 of the range of claims with the following characteristics. The modified base above acts as a relay for the other molecules or substances. The aforementioned other molecules or other substances themselves can be seen.

5. The modified DNA described in any of Claims 1 through 4 of the range of claims with the following characteristics. The aforementioned modified bases or, in some cases, the aforementioned relay molecules will be able to bond chemically with the associated molecules that are marked with enzymes. Or, marked with enzymes, they will be able to bond immunologically with antibodies having a selective affinity to the aforementioned modified bases or the aforementioned relay molecules.

6. The modified DNA described in any of Claims 1 through 5 of the range of claims with the following characteristics. In modified DNA in which at least one modified ribonucleotide base has bonded to at least one terminal, the aforementioned modified ribonucleotide base will be induced from ATP that has been modified by a modified base that had been covalently bonded at site 6, or preferably, site 8 of the adenine base. The bonding of the modified base described above takes place through a link type expressed with the following formula.

$$-NH-(CH_2)_X-X \text{ or } -CO-(CH_2)_X-X$$

Where, $_X$ is 2 – 20, or preferably, 6 – 12; X is a base that secures a bond with the base M selected from among bases that can undergo a bonding reaction with the chemical or immunological drug that has a selective affinity for the base M in question.

7. The modified DNA described above in Claim 6 of the range of claims with the following characteristics. The aforementioned link base CH_2 may undergo a partial substitution with CO or NH with the requirement that it not adjoin a base to which the substituent is equivalent.
8. The modified DNA described in Claims 1 through 7 of the range of claims with the following characteristics. The modified bases described above contain bases induced from biotin or avidin or 2, 4-dinitrophenyl.
9. A DNA modification method with the following characteristics. There must at least be ribonucleotides that are normally capable of conjugating in the DNA and the ribonucleotides that randomly carry the modified bases described in any of Claims 1 through 8 of the range of claims. Where necessary, the terminal DNA transferase is to be present, and the aforementioned DNA is processed using the ribonucleotides that carry the modified bases described in any of Claims 1 through 8 of the range of claims. After processing, when the bonding substance of the resulting DNA terminals is formed from modified nucleotide oligomers, the modified ribonucleotides of the aforementioned randomly occurring oligomers (other than the ribonucleotides that bonded directly with the terminal dioxyribonucleotide of the DNA terminal in question) are removed. It is preferred that an alkali base, especially sodium hydroxide, be used for this removal, under conditions that allow the separation of bonds that formed mutually between the ribonucleotides in the aforementioned oligomer segments.
10. The use of DNA modifications that allow the production of a restriction map of the aforementioned DNA by producing segments that all carry the same modified ribonucleotides as one of the terminals. Said segments are then recovered, demarcated and compared for size. Also used are the effect of the corresponding restriction enzymes and one modification of a DNA terminal using ribonucleotides that carry the modified bases described in any of ~~the modified bases described in any of~~ Claims 1 through 8 of the range of claims.
11. The use of DNA modification consisting of methods including the following. In order to analyze the arrangement of the DNA component nucleotides using the modification of one DNA terminal using ribonucleotides that contain modified bases like those described in any of Claims 1 through 8 of the range of claims, said DNA is processed with a chemical that can cause differential cleavage at the level of a type of base inside the aforementioned DNA is processed. The DNA segments are recovered and separated using a system that can demarcate them based on size. A reagent, that is able to bond with the molecules or substances that have selective affinity for the modified chemical bases of the terminal ribonucleotides and the chemical bases carried by the terminal ribonucleotides of the segments that carry terminal ribonucleotides, is used to cause the aforementioned DNA segment reaction and make it visible. Considering the characteristics of the chemicals used in cleavage at the level of each of the DNA segments, a step to determine the unmodified terminal nucleotides of each of the DNA segments was also included.

Detailed Description

A method of analyzing DNA segments such as those described above and DNA segments that have been marked using modified, ribonucleotides that can be identified using at least one terminal associated molecule.

This invention pertains to methods of analyzing arrangements of segments where at least one terminal is a modified nucleotide. More specifically, it pertains to methods of analyzing arrangements of segments like those described above as well as DNA segments that have been marked with modified ribonucleotide segments that can be identified using associated molecules.

Firstly, the Maxam & Gilbert Method (Proc. Natl. Acad. Sci. USA, Vol. 74, Edition. 2, pp. 560 – 564, February, 1977) can be listed as a technique for analyzing nucleotide sequences contained in DNA. In said method, there are different degrees of cleavage at the sites of the guanine and adenine bases as well as equivalent degrees of cleavage at the cytosine and thymine bases on the DNA to be analyzed. Lastly, a cleavage reaction is carried out so that cleavage takes place only at the site of the cytosine base. However, with this method, one of the terminals of the DNA being tested has to be marked in advance with a radioactive marker, generally ^{32}P. As described above, after the cleavage process, the segments produced are separated using a polyacrylamide gel electrophoresis method. This makes it possible to then reconstruct the structure of the various series of nucleotide chain sequences that made up the DNA to be tested initially. This sort of reconstruction generally performed using an autoradiographic image that forms on light-sensitive film that has been affixed to the gel.

It is well known that the technique of detecting the cleavage products with an autoradiogram on the gel plate is difficult. Because the radioactivity of the marked isotopes disperse into space in all directions, it is necessary to use ultra-thin plates in order to achieve sufficient resolution of the various electrophoresis bands in the gel. Generally, the thickness of said plates does not exceed 0.3 mm. As the gel plate becomes thicker, the resolution of the autoradiographic method probably degrades rapidly. Additionally, it cannot be said that there are no dangers associated with the radioactivity itself.

The purpose of this invention is to eliminate the shortcomings described above and particularly, to provide a method for modifying DNA that has sequences that are being studied. That would make the subsequent detection of DNA segments that had been obtained in the sequence analysis process easier. (The process of sequence analysis used could be the methods described above or any other method that allows the same sort of result to be achieved.)

The characteristics of the modified DNA of this invention include modified ribonucleotide oligomers or, preferably that a single modified ribonucleotide is bonded to at least 1 terminal of the DNA. The modification of the

ribonucleotide itself is done with chemical molecules that have been and bonded to said ribonucleotide covalently and identified. Said chemical molecules have at least 1 base that does not participate in this covalent bonding. Said base has a specific affinity to this base, so it recognizes the DNA to be modified and it can bond directly or indirectly to molecules or substances.

In particular, the aforementioned base itself can either be detected directly or, by bonding to another detectable substance, it could be used in a chemical or immunological affinity bond (related bond) with a molecule or substance that can be detected. Furthermore, under conditions where 1 or more ribonucleotides can bond with the DNA terminal and the DNA and the ribonucleotide come into contact with each other, the aforementioned base apparently does not allow change in the capacity of the ribonucleotides to bond to the DNA terminals in the presence of terminal DNA transferase.

Furthermore, as described above, this invention pertains to methods for producing modified DNA. With the methods of this invention, the DNA to be tested is processed using modified ribonucleotides as described above. This process is carried out in DNA and ribonucleotides that [illegible] and carry modified bases randomly. Normally, there are ribonucleotides that normally form pairs and, if necessary, terminal DNA transferase as well. When the DNA terminal bond substance that obtained after this process is made up of modified nucleotide oligomers, the modified ribonucleotides of the oligomers that are affected in the instance described above (when the ribonucleotides do not bond directly to the terminal deoxyribonucleotides of the DNA that [illegible], are removed. Preferably, this removal will take place using an alkali base, especially sodium hydroxide (soda), under conditions that allow the separation of bonds formed among the ribonucleotides in the aforementioned oligomer segments.

As described above, the process itself can produce DNA segments that are smaller, using known methods, from DNA that carries modified ribonucleotides on both terminals (or from DNA that carries them on 1 terminal). For this reason, either the effect of a chemical that can cause cleavages at the level of a specific nucleotide can be used or, preferably, the effect of a suitable restrictive enzyme, especially endonuclease would be used. However, in this case, the corresponding DNA must contain the corresponding restrictive sites.

Several of the aforementioned DNA segments are processed using restrictive enzymes and difference types of segments in which the same terminals have been marked are recovered, making it possible to create a restrictive map of the aforementioned DNA.

When the given restrictive enzyme responds to a single restrictive site, if the aforementioned DNA segments are processed using said enzyme, segments of a specific length can be produced. These segments can be used in the analyses of deoxyribonucleotides that make up the segments.

As described above, sequence analysis is one good use of this invention. The sequence analysis process of this invention includes the following steps. DNA that has 1 terminal marked with modified ribonucleotides is processed using a chemical capable of differential cleavages at certain types of bases of the DNA, especially at the base level described in the paper by Maxam & Gilbert mentioned above. The DNA segments are recovered and separated in a system that is capable of demarcating the DNA segments based on size. Of these segments, the modified chemical bases carried by the terminal ribonucleotides on segments having terminal ribonucleotides and reagents that bond to molecules or substances having selective affinity for said chemical bases, are reacted with the aforementioned DNA segments.

The modified ribonucleotides that bond to said DNA terminals are induced primarily from the following substances.

--- ~~5'~~ Adenosine - 5' - triphosphoric acid (ATP)
--- ~~5'~~ Guanosine - 5' - triphosphoric acid (GTP)
--- ~~5'~~ Cytidine - 5' - triphosphoric acid (CTP)
--- ~~5'~~ Uridine - 5' - triphosphoric acid (UTP)

The chemical bases that could bond covalently with the above ribonucleotides have many forms. However, they should have a base that allows bonding with an affinity substances that allows the detection of said chemical bases to be done with the naked eye. It is also required that they guarantee bonding between the modified ribonucleotides produced and the DNA terminals, and they must not interfere with the effect of the terminal DNA transferase.

Any chemical bases may be selected that can bond to the aforementioned ribonucleotide bases and can themselves be easily detected or, preferably other molecules or substances that can be detected and specifically recognized using visual methods.

As described above, other molecules and other substances are made up of enzymes whose existence can be detected by an effect that is produced in the presence of a base substance. It is desirable that the base substances be able to cause color gain or loss reactions. Or in broader applications, that they produce variations in absorbed spectra that can be detected using colorimetry or spectrometry. For example, the following substances may certainly be used: molecules or substances that produce fluorescent reactions or variations in optical concentrations, or those that induce bases from such substances as aminofluorene, dansyl chloride or rhodamine.

Suitable modified bases include those chemical bases that have been demonstrated to have affinity with chemical molecules of a separate type. These modified chemical bases include for example, biotin or avidin. It is known that these molecules have mutual affinity and bases induced from one of the molecules performs the function of modifying select

ribonucleotides. The other molecule is either capable of bonding with a reagent that has been marked with an enzyme or is capable of bonding to an enzyme. In this case, the conditions

described in French Patent Application 7810975 of the Pasteur Institute, submitted on April 13th, 1978 could be used. The aforementioned reagents could be made from molecules having specific affinity with modified bases or specific antibodies to modified bases.

Moreover, other useful bases of starting-point ribonucleotides include antigens or haptens. The antigens or haptens could be recognized using antibodies formed against those antigens or haptens ahead of time, when the blood serum albumin or polypetides such as polylysine are bonded to a macromolecular carrier in advance. These antigens and haptens include, biotin and avidin themselves, acetyl aminofluorene, peptides, hormones and prostaglandins, especially prostaglandins and lectins that correspond to specific antiserum or antigens. Among enzymes that make lectins detectable, it is known that lectins have the capacity to bond, especially to peroxidase and beta-galactosidase. The blood serum and antibodies described above are commercially available.

Be that as it may, however, depending upon circumstances, the molecules or substances that have an affinity for the modified bases described above, may serve only to function as relays for the other molecules or other substances that themselves become visible under the aforementioned conditions. For example, substances that have affinity for the modified bases described above are made from antibodies that can be recognized using the corresponding antibodies that aren't themselves marked. The corresponding antibodies themselves can be bonded to enzymes that can't act on specific base substances under conventional conditions involving to immunoenzymological quantification.

Generally, the modified bases of ribonucleotides can be any chemical molecules or chemical substances can bond to ribonucleotides and then be detected under the conditions described above. However, they must not interfere with the capacities of the nucleotides to be modified (which bond to the DNA terminals under the effect of DNA terminal transferase). This characteristic also serves as the basis for the identification test. That is, during the identification test, specific DNA segments that carry on at least 1 terminal, the ribonucleotides that are modified using molecules or substances to be detected, become manifest, that is, they can become effectively visible, especially under the conditions named above. However, at this point, the phenotypically changed DNA must be formed under the conditions for normal bonding of an unmodified identical ribonucleotide or oligomer of said ribonucleotide to at least 1 terminal of said DNA segment. In the presence of the DNA terminal transferase, the corresponding DNA segment and the modified ribonucleotides must react with each other.

When the starting-point ribonucleotides are formed from ATP, the aforementioned modified chemical bases bond to site 6 or site 8 of the adenine base.

It is advantageous to have the aforementioned bond formed through the link shown in the formula below.

- $NH – (CH_2)_X – X$ or

- $NH – (CH_2)_X – X$

(Where $_X$ is 2 to 20 (especially 6 – 12) and X is a base that can secure a bond with the base M that is selected from among bases that can undergo a bonding reaction with chemical or immunological pharmaceuticals having selective affinity for said base M.)

The base CH_2 can be partially substituted using base CO or NH in the aforementioned link. Naturally, of course, the same bases must not be in contact with each other for these substitution bases

For example, when site 8 of the adenine base of ATP is modified, the modified ribonucleotides that are produced can be expressed using the formula below. (Where the link is a $– NH – (CH_2)_X – X –$ type.)

[See original for chemical formula] Formula I

In the formula, R is a triphosphate base, while $_X$, X and M are the same as above. It is advantageous to have the base X made from the base NH or CO.

For example, in order to manufacture an inducer from ATP, to which bromine has been added in advance to the site 8, using formula I, a reaction would be brought about under suitable conditions with the ATP described above and the compound from the formula $H_2N – (CH_2)_X – X – Y$. Next, base Y is substituted using base M, described above. This substitution is performed using a condensation reaction with the MZ molecule. During said reaction, the condensing product from formula I is formed while the molecule Y – Z is liberated.

When base X is NH, it is preferable to have hydrogen as Y. When X is CO, it is preferable to have a hydroxyl as Y. In the

condensation reaction described above, Z could be any base that can break away from M. For example, when the chemical molecule that is the donor of the desired base is 1 – fluoro – 2, 4 – dinitro ~~-2, 4~~ benzene, this would be fluorine. When it is a peptide, it would be a hydroxyl or hydrogen. In the latter case, in order to bond the peptide to the terminal of the aforementioned link, a condensation reaction is used when base XY is made of base NH2 or COOH. This reaction has been used in protein chemistry for some time and takes place between the amine base and the carboxyl base that are carried by two separate peptide elements that are to be bonded. The bonding reaction can be carried out, for example by using condensing in the presence of a condensing agent such as dicyclohexyl-carbodiimide or by using condensation after preforming an activated ester on a carboxyl function group that is carried by 1 of two peptide elements.

It is known that site 8 on the adenine ring of the ATP is not the only place that can bond with chains containing modified bases such as those described above. For example, it is possible to substitute one of the hydrogen atoms carried by site 6 of the adenine ring using a chain that has a modified base. Or, for example, a reaction is brought about between ATP and iodine acetate, which can form a quaternary salt in advance where the nitrogen of site 1 intervenes, or an equivalent organic iodide. Then, after a period of sufficient time (such as 72 hours) in a basic medium that is a mildly basic pH, especially a pH of 8, the aforementioned base can be heated to a temperature of 35°C and modified into a substitution product. In this substitution product, ~~one of the hydrogen atoms of NH2 base~~ one of the hydrogen atoms of the NH2 base, which is bonded to the carbon atom at site 6 of the adenine base, is substituted. (This reaction is known by the name "Dimroth translocation.")

The results (when organic iodide comes from the iodine acetate) of the above produce the compounds shown in formula II below.

[See original for chemical formula] Formula II

Next, this compound is converted by reacting with the compound in the formula above ($H_2N - (CH_2)_X - X - Y$). The reaction conditions used allow the chemical bonding of the carbonyl group that is initially contained in the carboxyl group in the compound in Formula II and the imino group that belongs initially to the amino function group compound $H_2N - (CH_2)_X - X - Y$. The compound $H_2N - (CH_2)_X - X - Y$ itself can bond with the compounds in formula MZ under the conditions described above.

Naturally, all of the preceding descriptions describe specific preparation methods for bonding 1 modified base, selected from among modified bases corresponding to affinity molecules as described above, with ATP.

The GTP inducer is manufactured using the same sort of method. In the case, the aforementioned modified chemical base is bonded to site 2 or preferably site 8 of the guanine base of the GTP under the same sorts of conditions. Normally, the same reaction mechanisms can be applied.

In the same way, UTP or CTP could also be put through the previous applications, using the chemical base that corresponds to the aforementioned conditions. However in these cases, the methods used are completely different from those described in the paper by P. R. Langer et al (Proc. Natl. Acad. Sci. USA, Vol. 78, Edition 11, pp. 6644 – 6647, November 1981).

Other characteristics of this invention should be clear from the descriptions of desirable embodiments of this invention.

Manufacturing 8 – [- N – (dinitro-phenyl) – amino – hexyl] – amino adenosine 5' – triphosphate

8 – (aminohexyl) – amino adenosine 5' – triphosphate and fluoro – 1 – dinitro – 2, 4 – benzene are caused to react in the presence of magnesium salts, especially magnesium chloride, at a temperature of 40°C, in a 10:1 volume percent mixture of water and ethanol with a pH of 8.8. The final reaction products in the following formula are produced.

[See original for chemical formula] Formula III

The inducer in Formula II shall be referred to hereafter as ATP-DNP. This inducer is recovered after a purification process including filtration using a Sephadex G50 type molecular sieve and elution using a slope from 0.2 N LiCl (pH 5.5) up to 0.5 N LiCl (pH 2) and anchoring ribonucleotides onto DEAE cellulose. The reaction recovery rate was 52%. The gathered fractions were analyzed using radiation-absorbing spectrometry with wavelengths of 280 and 360 nm, respectively. Fractions having optical concentration ratios (DO_{280}/DO_{360}) equivalent to 4 in the 2 wavelength regions described above, were collected. For the products contained in said fractions, 1

mol of DNP was bonded with 1 mol of ATP. Furthermore, there was only one staining point in the thin-film chromatography system for said fractions. The products of said fractions were freeze-dried.

One characteristic of said products is that they could be recognized by antibodies to the dinitro 2, 4- benzene that had bonded in advance to the blood serum albumin macromolecular carrier, which had been formed in advance. This type of antibody is also commercially available.

Manufacturing 8 – (N – biotinyl-amino hexyl) – amino adenosine – 5' – triphosphate

8 – (amino hexyl) – amino adenosine – 5' – triphosphate and biotinyl – N – hydroxy-succinimide ester was condensed under the conditions described by Langer et al, that were used when manufacturing biotinyl-UTP from 5 – (3 – amino) allyl uridine. This produced the compounds in the formula below.

[See original for chemical formula] Formula IV

Manufacturing DNA whose Terminals are Marked with Modified Ribonucleotides

600 u mols of ATP-DNP and 10 ug of DNA segments containing 500 base pairs were reacted for 24 hours at 37°C in a buffering solution in the presence of 30 units of DNA-terminal-transferase. The buffering solution had the composition shown below (final amount: 200 ul).

100 mM potassium cacodylate
1 mg/ml bovine blood serum albumin
1 mM dithiothreitol
1 mM cobalt chloride

Passing the solution through a commercially available Sephadex G50(R) molecular sieve column, we purified DNA that had ATP-DNP bases on the terminals.

One drop of said fraction was placed on a cellulose filter. The filter was dried and brought into contact with a rabbit anti-DNP antibody solution. The antibodies that did not bond were washed away. Next, the filter was brought into contact with a rabbit antibody solution that had bonded with peroxidase. The antibodies that did not bond were washed away and the existence of antibodies that had bonded to the filter in the peroxidase base solution was detected. Said solution was made up of the following.

10 ul of oxygenated water H_2O_2 (110x solution)
9.5 ml potassium acetate, 0.05 M pH 5.1
2 mg carbazole (05 ml N - N' – dimethylformamide)

This was used to detect the presence of rabbit anti-antibodies in the filtered product using the formation of a red amber precipitate.

The sensitivity of the method was such that extreme trace amounts of DNA (ie 380 x 10^{-4} picomols of DNA) could be detected.

The application of the modified DNA of this invention to the analysis of constituent nucleotide sequences in DNA.

The first step in the method of analyzing the nucleotide sequences contained within the double stranded DNA has been summarized as follows.

[See original for chemical formulae]

(1)

Terminal Transferase + ATP-DNP

(2)

(3)

Restriction Enzymes

For the starting point DNA (1), the terminals of each chain are indicated using two parallel lines and the symbols 3' and 5'.

Reactions with ATP-DNP in the presence of DNA-terminal-transferase produced the modified DNA segments shown in (2). The AMP-DNP oligomer is bonded to the 3' terminals of said segments. (In the example in the diagram, each oligomer has three base units of AMP-DNP. The AMP is the ATP-DMP inducer element with 2 phosphate bases per ATP-DMP monomer removed due to oligomerization.)

The DNA that was modified as above was subjected to alkali hydrolysis in a 1M sodium hydroxide solution at 40°C. This made is possible to once again recover the modified DNA containing each of the sole modified ribonucleotide on terminals using a molecular sieve.

For example, using the effect of the restriction enzyme under the conditions described in Maxam & Gilbert, we obtained the two segments indicated in (4) with the symbols A and B. It is clear that the process order for (2), (3) and (4) can be changed as desired.

For example, after isolating and purifying the segment B, it was divided into several lots and each lot was subjected to various differential cleavage reactions under the conditions described by Maxam & Gilbert. After these selective cleavage reactions, the product was processed using acrylamide gel electrophoresis under the conditions described by the same authors. Each of the DNA segments that had a marked terminal was separated into bands based on size.

This invention allows a variety of marked segments that are to be, for example, detected in situ using a gel, to be brought into contact with antibody solutions under the conditions described above. Additionally, affixing a cellulose filter or the same sort of carrier to the gel makes it possible to transfer at least a portion of the respective electrophoresis bands to said filter, allowing their detection, under the conditions named above, for example, on the filter itself.

By using the extremely sensitive method of this invention, the fraction bands can be detected visually and directly on the filter or in the gel, which means that the traditional ultra thin gel plates don't have to be used.

When the modified ribonucleotides are new, this invention naturally includes the modified ribonucleotides themselves. In such cases, under the aforementioned conditions, the modified ribonucleotides may be induced from ATPs.

Naturally, as is clear from the preceding, this invention is not restricted to the particular examples, uses or embodiments shown here. Conversely, it includes all variations.

International Search Report

[see original for English]